DEVELOPMENT OF OPTO-CHEMICAL MICROSCOPE SYSTEM FOR SPATIO-TEMPORAL ANALYSIS OF SIGNALS IN SELF-ORGANIZED NEURONS

Takashi Sakurai[1,3], Hidenori Taki[2], Junpei Nishimoto[2], Kazuhiro Takahashi[2,3], Makoto Ishida[1,2,3], Koichi Okumura[2,3], Kazuaki Sawada[2,3]

[1]Electronics-Inspired Interdisciplinary Research Institute, and [2]Department of Electrical and Electronic Information Engineering, Toyohashi University of Technology, Japan, [3]JST CREST, Japan

ABSTRACT

To study dynamics of molecular signals in the living neurons, we combined an inverted fluorescence microscope with a probe-type ion image sensor. By the newly developed opto-chemical microscopy, the changes in both $[pH]_o$ and $[Ca^{2+}]_i$ of neurons were simultaneously visible among hippocampal slices in culture. Immediately after depolarization, both cellular signals showed significant changes, and the peak amplitude of the signal was dependent on the site of neuronal cells. We established in situ assessment of both morphology and function of neuronal networks by the present ion image sensor-based opto-chemical microscope system.

KEYWORDS

ion image sensor, pH, hippocampus, Ca, fluorescence microscope

INTRODUCTION

By using CCD/CMOS image sensor technique, ion image sensor was developed for the 2D display of pH [1]. It produces an electrical output in proportion to the surface potential on each pixel, thus displays the concentration of extracellular pH ($[pH]_o$) with a high spatial resolution (~100 μm). With the chemical microsensor, 2D imaging of not only $[pH]_o$ but also transmitter releases were possible in the living cells without labeling [2]. We have used this label-free technique for the detection of proton in the acid secreting cells, osteoclasts and gastric glands [3]. To visualize a release of neuro-signals in the living neurons, we have developed the probe-type ion image sensor module (p-ion-IS), and combined it with fluorescence microscope (FM) for spatio-temporal analysis of neuronal functions in the brain.

COMPONENTS OF OPTO-CHEMICAL NEUROIMAGING SYSTEM

The elemental components for the construction of the opto-chemical neuro-imaging system are shown in Figure 1. The sensing area (SA) locating at the bottom of p-ion-IS is 4.16 x 4.16 mm^2 square with 32 x 32 pixels. By the manipulator control of p-ion-IS, SA was directly contacted with surface of the brain samples. The hippocampal slices were prepared from the rat brain [4], cultured for 1 week, and put on the window part of a cell chamber. To obtain the images at a higher S/N ratio in the whole field of SA, we used a macro-objective lens equipped inverted FM. The high NA objective was capable of resolving the CA1 region where glutamate-sensitive neurons were aligned.

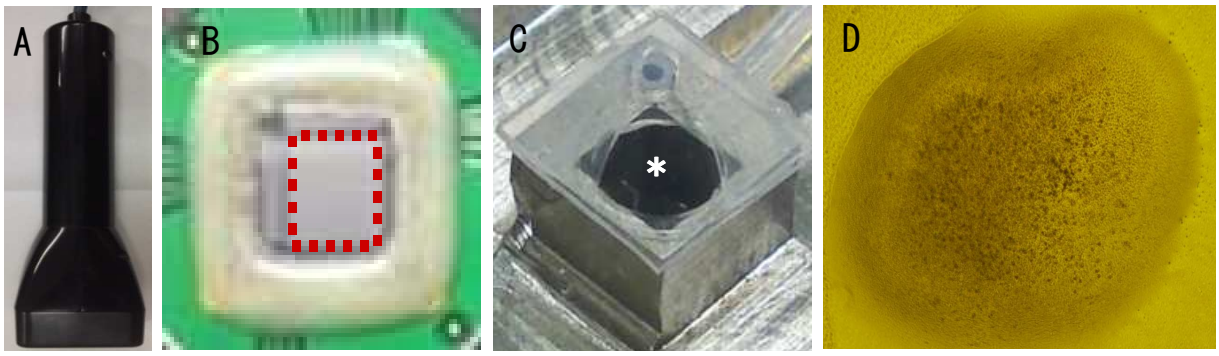

Figure 1. Photographs of p-ion-IS (A), SA (dashed lines in B), cell chamber (C) and brain slice (D). Cultured brain slice was put on the window (asterisk in C) of cell chamber.

OPTO-CHEMICAL MICROSCOPE SYSTEM AND CELL STIMULATIONS

The dual imaging system for both $[pH]_o$ and intracellular concencentration of Ca^{2+} ($[Ca^{2+}]_i$) is illustrated in Figure 2. After loading Ca-sensitive fluorescent dyes (fluo4AM), cultured slice was superfused with the circulating medium through inlet/outlet tubes. We measured the cell responses of $[pH]_o$ by p-ion-IS, and $[Ca^{2+}]_i$ by FM, and stimulated cells with chemicals added through the inlet tube. Fluo4 was excited with 480 nm light, and the emitted green fluorescence was recorded with CCD camera. Before the recording, slices were kept in the recording medium containing 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM D-glucose, and 10 mM HEPES (pH=7.2 with NaOH).

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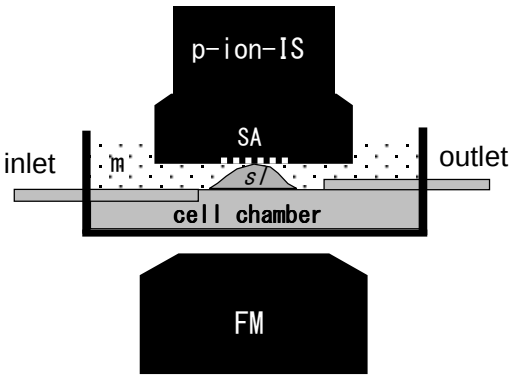

Figure 2. Schematic diagram of opto-chemical microscope system consisting p-ion-IS, cell chamber and FM. sl; slice preparation putting on the cell chamber. m; medium.

DUAL IMAGING OF $[pH]_o$ and $[Ca^{2+}]_i$ IN THE BRAIN SLICE

The sequential images of $[pH]_o$ and $[Ca^{2+}]_i$ in the hippocampal slices were recorded at the 4 mm^2 square. The $[pH]_o$ was slightly lower in the corresponding area where the slice images were visible by FM. Before stimulation, no significant changes were observed in both signals. After stimulation with chemicals adding through the inlet tubes, $[Ca^{2+}]_i$ was changed in the whole region of slice (Fig. 3B), while $[pH]_o$ change was partial (Fig. 3A). Between the CA3 and CA1 regions, $[pH]_o$ showed significant changes in 1 min after the stimulation.

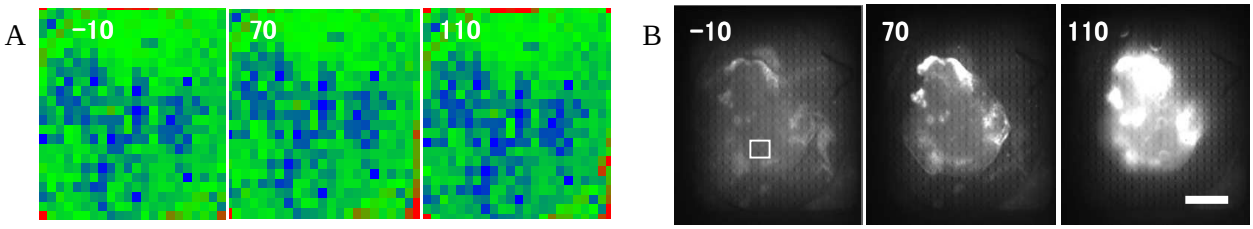

Figure 3. Sequential images simultaneously recorded by using p-ion-IS (A; $[pH]_o$) and FM (B; $[Ca^{2+}]i$). Numbers indicate the time (sec) after the cell stimulation. Bar:1 mm.

TIME COURSE OF OF $[pH]_o$ and $[Ca^{2+}]_i$

Figure 4 shows the time course of the cell signals in the area of a square indicated in Fig. 3B. The averaged $[pH]_o$ before stimulation was about 7.2 almost same as the recording medium. Within 1 min after stimulation with an ethanol (up to 1 mM), the $[pH]_o$ decreased, while $[Ca^{2+}]_i$ increased. In average, the degree of the $[pH]_o$ decrease was 0.75, and $[Ca^{2+}]_i$ increase was 25% compared to the basal level of fluorescence intensity. Both signals did not recovered to the initial level in a few minutes. The spatio-temporal analysis of Ca-dependent proton release from a neuron was possible by the opto-chemical microscopy. The sensitivity to the potent stimulants to neuron was clearly distinguishable by the analysis of $[pH]_o$ measurement using the ion image sensor.

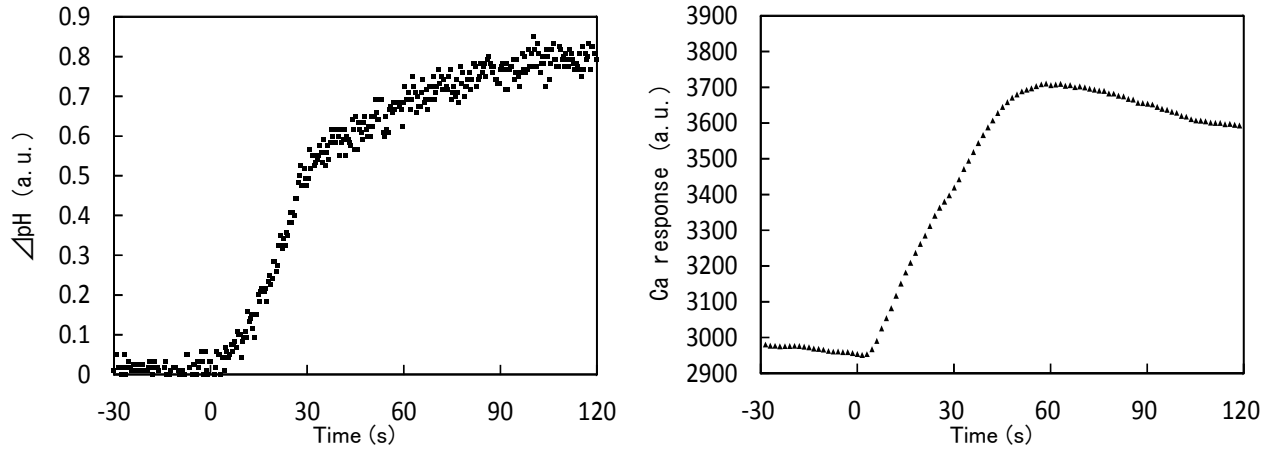

Figure 4. Changes in $[pH]_o$ (left) and $[Ca^{2+}]_i$ (right) simultaneously recorded by the opto-chemical microscopy. $[pH]_o$ change wascalculated as the difference to the initial value, and Ca response was indicated as relative fluorescence intensities of fluo4. Time indicates after cell stimulations.

SELF-ORGANIZATION OF THE NEURONS

To extend the study of *in situ* analysis for the neural-signals, we prepared a neuronal network model artificially constructed on the polydimethylsiloxane (PDMS) chamber. As shown in Figure 5, extension of nerve fiber and formation of synapse-like structures were induced after 1 week culture of neurons which was dissociated from hippocamus. The neural outgrowth appeared not only in the bottom of circled area (diameter=200 μm), but also in the flow path of the 10 μm width fabricated inside the PDMS chamber. The fiber protrusion was over 200 μm length, and sometimes formed a synapse at the terminal. These results indicate the culture of dissociated neurons in the PDMS chamber is useful for induction of the self-organization and functional analysis of neurons.

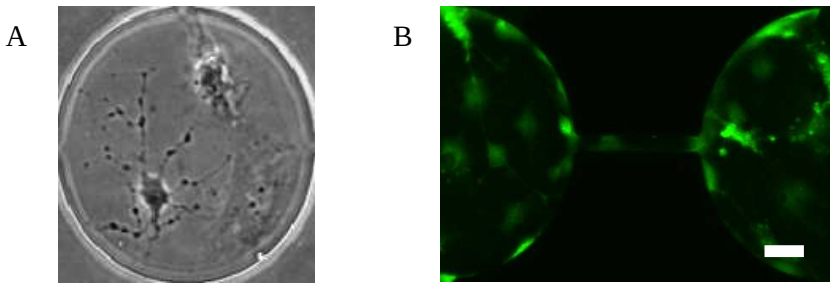

Figure 5. Morphology of neurons cultured in a PDMS chamber for 1 week.
Left, phase contrast image. Right, fluorescence image of cells labeled with FITC. Bar, 20 μm.

CONCLUSION

For the monitoring of neuronal signals in the brain slice, we successfully combined a probe-type ion image sensor with fluorescence microscope. By using the opto-chemical microscope, changes in $[pH]_o$ and $[Ca^{2+}]_i$ of the slice were visible with time. We established in situ realtime assessment of both morphology and function of neuronal networks, and the spatio-temporal analysis of cellular signals both inside and outside of the living cells.

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CONTACT

Takashi Sakurai sakurai@eiiris.tut.ac.jp